IDENTIFICATION AND CHARACTERIZATION OF MULTIPLE SPLICE VARIANTS OF THE KAPPA$_3$-RELATED OPIOID RECEPTOR (KOR-3) GENE

CROSS-REFERENCE TO RELATED APPLICATIONS

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STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH

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TECHNICAL FIELD

The present invention relates to kappa$_3$-related-opioid receptor-3 (KOR-3) splice variant polypeptides, to KOR-3 splice variant polynucleotides, to methods of screening compositions for agonists and antagonists of the splice variant receptor activities and to methods of measuring splice variant polypeptide binding activities.

BACKGROUND ART

Opiates are drugs derived from opium and include morphine, codeine and a wide variety of semisynthetic opioid congeners derived from them and from thebaine, another component of opium. Opioids include the opiates and all agonists and antagonists with morphine-like activity and naturally occurring endogenous and synthetic opioid peptides. Morphine and other morphine-like opioid agonists are commonly used pharmaceutically to produce analgesia.

There are now many compounds with pharmacological properties similar to those produced by morphine, but none has proven to be clinically superior in relieving pain. References to morphine herein will be understood to include morphine-like agonists as well. The effects of morphine on human beings are

relatively diverse and include analgesia, drowsiness, changes in mood, respiratory depression, decreased gastrointestinal motility, nausea, vomiting, and alterations of the endocrine and autonomic nervous systems. Pasternak (1993) Clin. Neuropharmacol. 16:1. Doses of morphine need to be tailored based on individual sensitivity to the drug and the pain-sparing needs of the individual. For instance, the typical initial dose of morphine (10mg/70kg) relieves post-operative pain satisfactorily in only two-thirds of patients. Likewise, responses of an individual patient may vary dramatically with different morphine-like drugs and patients may have side effects with one such drug and not another. For example, it is known that some patients who are unable to tolerate morphine may have no problems with an equianalgesic dose of methadone. The mechanisms underlying variations in individual responses to morphine and morphine-like agonists have not been defined.

The analgesic effects of morphine are transduced through opioid receptors in the central nervous system (CNS), located at both spinal and multiple supraspinal sites. Morphine and other agonists induce profound analgesia when administered intrathecally or instilled locally into the dorsal horn of the spinal cord. Several mechanisms of action are believed to mediate the inhibition of nociceptive reflexes from reaching higher centers of the brain, including the inhibition of neurotransmitter release by opioid receptors on the termini of primary afferent nerves and post synaptic inhibitory actions on interneurons and on the out-put neurons of the spinothalamic tract.

Profound analgesia can also be produced by the instillation of morphine into the third ventricle or within various sites in the midbrain and medulla, most notably the periaqueductal gray matter, the nucleus raphe magnus, and the locus ceruleus. Although the neuronal circuitry responsible has not been defined, these actions produce enhanced activity in the descending aminergic bulbospinal pathways that exert inhibitory effects on the processing of nociceptive information in the spinal cord. Simultaneous administration of morphine at both spinal and supraspinal sites results in a synergized analgesic response, with a ten-fold

reduction in the total dose of morphine necessary to produce equivalent analgesia at either site alone.

Morphine also exerts effects on the neuroendocrine system. Morphine acts in the hypothalamus to inhibit the release of gonadotropin releasing hormone (GnRH) and corticotropin-releasing factor (CRF), thus decreasing circulating concentrations of luteinizing hormone (LH), follicle stimulating hormone (FSH), and adrenocorticotropin (ACTH), and β-endorphin. As a result of the decreased concentrations of pituitary trophic hormones, the concentrations of testosterone and cortisol in the plasma decline. The administration of opiates increases the concentration of prolactin (PRL) in plasma, most likely by reducing the dopaminergic inhibition of PRL secretion. With chronic administration, tolerance eventually develops to the effects of morphine on hypothalamic releasing factors.

Opiates can interfere with normal gastrointestinal functioning. Morphine decreases both gastric motility and the secretion of hydrochloric acid in the stomach. Morphine may delay passage of gastric contents through the duodenum for as long as 12 hours. Morphine also decreases biliary, pancreatic, and intestinal secretions and delays the digestion of food in the small intestine. Propulsive peristaltic waves in the colon are diminished or abolished after administration of morphine and commonly, constipation occurs. For a detailed review of the physiologic effects of morphine, see Reisine and Pasternak (1996) Goodman & Gilman's The pharmacological basis of therapeutics, Ninth Edition (Hardman et al. eds.) McGraw-Hill pp 521-555.

Morphine also exerts effects on the immune system. The most firmly established effect of morphine is its ability to inhibit the formation of rosettes by human lymphocytes. The administration of morphine to animals causes suppression of the cytotoxic activity of natural killer cells and enhances the growth of implanted tumors. These effects appear to be mediated by actions within the CNS. By contrast, β-endorphin enhances the cytotoxic activity of human monocytes in vitro and increases the recruitment of precursor cells into the killer cell population; this peptide also can exert a potent chemotactic effect on these cells. A novel type of receptor (designated ∈) may be involved. These

effects, combined with the synthesis of proopiomelanocortin (POMC) and preproenkephalin by various cells of the immune system, have stimulated studies of the potential role of opioids in the regulation of immune function. Sibinga and Goldstein (1988) Annu. Rev. Immunol. 6:219.

Side effects resulting from the use of morphine range from mild to life-threatening. Morphine causes constriction of the pupil by an excitatory action on the parasympathetic nerve innervating the pupil. Morphine depresses the cough reflex through inhibitory effects on the cough centers in the medulla. Nausea and vomiting occur in some individuals through direct stimulation of the chemoreceptor trigger zone for emesis, in the postrema of the medulla. Therapeutic doses of morphine also result in peripheral vasodilatation, reduced peripheral resistance and an inhibition of baroreceptor reflexes in the cardiovascular system. Additionally, morphine provokes the release of histamines, which can cause hypotension. Morphine depresses respiration, at least in part by direct effects on the brainstem regulatory systems. In humans, death from morphine poisoning is nearly always due to respiratory arrest. Opioid antagonists can produce a dramatic reversal of severe respiratory depression and naloxone is currently the treatment of choice. High doses of morphine and related opioids can produce convulsions, which are not always relieved by anticonvulsant agents, such as naloxone.

The development of tolerance and physical dependence with repeated use is a characteristic feature of all opiates. Dependence seems to be closely related to tolerance, since treatments that block tolerance to morphine also block dependence. In vivo studies in animal models demonstrate the importance of neurotransmitters and their interactions with opioid pathways in the development of tolerance to morphine. Blockade of glutamate actions by noncompetitive and competitive NMDA (N-methyl-D-aspartate) antagonists blocks morphine tolerance. Trujillo and Akil (1991) Science 251:85; and Elliott et al. (1994) Pain 56:69. Blockade of the glycine regulatory site on NMDA receptors has similar effects to block tolerance. Kolesnikov et al. (1994) Life Sci. 55:1393. Administering inhibitors of nitric oxide synthase in morphine-tolerant animals

reverses tolerance, despite continued opioid administration. Kolesnikov et al. (1993) Proc. Natl. Acad. Sci. U.S.A. 90:5162. These studies indicate several important aspects of tolerance and dependence. First, the selective actions of drugs on tolerance and dependence demonstrate that analgesia can be dissociated from these two unwanted actions. Second, the reversal of preexisting tolerance by NMDA antagonists and nitric oxide synthase inhibitors indicates that tolerance is a balance between activation of processes and reversal of those processes. These observations suggest that, by use of selective agonists or antagonists, tolerance and dependence in the clinical management of pain can be minimized or disassociated from the therapeutic effects.

In addition to morphine, there are a variety of opioids suitable for clinical use. These include, but are not limited to, Levorphanol, Meperidine, Fentanyl, Methadone, Codeine, Propoxyphene and various opioid peptides. Certain opioids are mixed agonists/antagonists and partial agonists. These include pentazocine, nalbuphine, butorphanol, and buprenorphine. The pharmacological effects of levorphanol closely parallel those of morphine although clinical reports suggest that levorphanol produces less nausea.

Meperidine exerts its chief pharmacological effects on the CNS and the neural elements in the bowel. Meperidine produces a pattern of effects similar but not identical to those described for morphine. In equianalgesic doses, meperidine produces as much sedation, respiratory depression, and euphoria as morphine. The pattern of unwanted side effects that follow the use of meperidine are similar to those observed after equianalgesic doses of morphine, except that constipation and urinary retention are less common.

Fentanyl is a synthetic opioid estimated to be 80 times as potent as morphine as an analgesic. High doses of fentanyl can result in severe toxicity and produce side effects including muscular rigidity and respiratory depression.

Methadone is an opioid with pharmacological properties similar to morphine. The properties of methadone include effective analgesic activity, efficacy by the oral route and persistent effects with repeated administration. Side effects include detection of miotic and respiratory-depressant effects for more

than 24 hours after a single dose, and marked sedation is seen in some patients. Effects on cough, bowel motility, biliary tone and the secretion of pituitary hormones are qualitatively similar to those of morphine. In contrast to morphine, codeine is approximately 60% as effective orally as parenterally, both as an analgesic and as a respiratory depressant.

Codeine has an exceptionally low affinity for opioid receptors, and the analgesic effect of codeine is due to its conversion to morphine. However, codeine's antitussive actions probably involve distinct receptors that bind codeine specifically.

Propoxyphene produces analgesia and other CNS effects that are similar to those seen with morphine. It is likely that at equianalgesic doses the incidence of side effects such as nausea, anorexia, constipation, abdominal pain, and drowsiness would be similar to those of codeine.

Opioid antagonists have therapeutic utility in the treatment of overdosage with opioids. As understanding of the role of endogenous opioid systems in pathophysiologic states increases, additional therapeutic indications for these antagonists will emerge. If endogenous opioid systems have not been activated, the pharmacological actions of opioid antagonists depend on whether or not an opioid agonist has been administered previously, the pharmacological profile of that opioid and the degree to which physical dependence on an opioid has developed. The antagonist naloxone produces no discernible subjective effects aside from slight drowsiness. Naltrexone functions similarly, but with higher oral efficacy and a longer duration of action. Currently, naloxone and naltrexone are used clinically to treat opioid overdoses. Their potential utility in the treatment of shock, stroke, spinal cord and brain trauma, and other disorders that may involve mobilization of endogenous opioids remains to be established.

The complex interactions of morphine and drugs with mixed agonist/antagonist properties are mediated by multiple classes of opioid receptors. Opioid receptors comprise a family of cell surface proteins, which control a range of biological responses, including pain perception, modulation of affective behavior and motor control, autonomic nervous system regulation and

neuroendocrinological function. There are three major classes of opioid receptors in the CNS, designated mu, kappa and delta, which differ in their affinity for various opioid ligands and in their cellular distribution. The different classes of opioid receptors are believed to serve different physiologic functions. Olson et al. (1989) Peptides 10:1253; Lutz and Pfister (1992) J. Receptor Res. 12:267; and Simon (1991) Medicinal Res. Rev. 11:357. Morphine produces analgesia primarily through the mu-opioid receptor. However, among the opioid receptors, there is substantial overlap of function as well as of cellular distribution.

Members of each known class of opioid receptor have been cloned from human cDNA and their predicted amino acid sequences have been determined. Yasuda et al. (1993) Proc. Natl. Acad. Sci. U.S.A. 90:6736; and Chen et al. (1993) Mol. Pharmacol. 44:8. The opioid receptors belong to a class of transmembrane spanning receptors known as G-protein coupled receptors. G-proteins consist of three tightly associated subunits, alpha, beta and gamma (1:1:1) in order of decreasing mass. Following agonist binding to the receptor, a conformational change is transmitted to the G-protein, which causes the G-alpha subunit to exchange a bound GDP for GTP and to dissociate from the beta and gamma subunits. The GTP-bound form of the alpha subunit is typically the effector-modulating moiety. Signal amplification results from the ability of a single receptor to activate many G-protein molecules, and from the stimulation by G-alpha-GTP of many catalytic cycles of the effector.

Most opioid receptor-mediated functions appear to be mediated through G-protein interactions. Standifer and Pasternak (1997) Cell Signal. 9:237. Antisense oligodeoxynucleotides directed against various G-protein alpha subunits were shown to differentially block the analgesic actions of the mu-, delta-, and kappa- opioid-agonists in mice. Standifer et al. (1996) Mol. Pharmacol. 50:293.

The amino acid sequences of the opioid receptors are approximately 65% identical, and they have little sequence similarity to other G-protein-coupled receptors except for somatostatin. Reisine and Bell (1993) Trends Neurosci. 16:506. The regions of highest similarity in sequence are the sequences predicted

to lie in the seven transmembrane-spanning regions and the intracellular loops. Regions of amino acid sequence divergence are the amino and carboxy termini and the second and third extracellular loops.

Each receptor subtype has a characteristic pattern of expression. Mu-opioid receptor mRNA is present in the periaqueductal gray, spinal trigeminal nucleus, cuneate and gracile nuclei, and thalamus regions of the brain involved in pain perception and associated with morphine analgesia (Defts et al. (1994) J. Comp. Neurol. 345:46); in nuclei involved in control of respiration, consistent with the ability of morphine to depress respiration; and in neurons of the area postrema, where morphine has been shown to cause nausea and induce vomiting. Other consequences of mu-opioid receptor activation include miosis, reduced gastrointestinal motility, and feelings of well-being or euphoria. Pasternak (1993). The pattern of mu-opioid receptor mRNA expression correlates with the brain centers involved in mediating the biological actions of morphine and mu-selective agonists. Delta-opioid receptor mRNA is found in the dorsal horn of the spinal cord. Kappa$_1$-opioid receptor mRNA is expressed in the hypothalamic regions, which may account for many of the neuroendocrine effects of the kappa selective agonists.

Alternative splicing has been observed with a number of G-protein-coupled receptors, including somatostatin 2 (Vanetti et al. (1998) FEBS Lett. 311:290), dopamine D2 (Guiramand et al. (1995) J. Biol. Chem. 270:7354), prostaglandin EP3 (Namba et al. (1993) Trends Pharmacol. Sci. 16:246), serotonin receptor subtypes 5-HT$_4$ and 5-HT$_7$ (Lucas and Hen. (1995) Trends Pharmacol. Sci. 16:246) and KOR-3. Bare et al. (1994) FEBS Lett. 354:213; and Zimprich et al. (1995) FEBS Lett. 359:142.

Several opioid receptor splice variants have been identified and characterized. At least two MOR-1 splice variants are known, the human MOR-1A and the rat MOR-1BI. Bare et al. (1994); and Zimprich et al. (1995). The hMOR-1A splice variant consists of exons 1, 2, 3 and a new exon 3a, and was determined to possess ligand binding characteristics similar to the full-length MOR-1. Bare et al. (1994).

In the case of the Kappa-opioid receptor, few variants have been found. This member of the opioid receptor family was cloned from human (ORL-1; hereinafter hKOR-3), mouse (KOR-3; hereinafter mKOR-3) and other species. U.S. Patent No. 5,747,279; Bunzow et al. (1994) FEBS Lett. 347:284-288; Fukuda et al. (1994) FEBS Lett. 343:42-46; Wick et al. (1994) Molec. Brain Res. 27:37-44; Pan et al. (1996) Gene 171:255-260; Mollereau et al. (1994) FEBS Lett. 341:33-38; and Pan et al. (1995) Mol. Pharmacol. 47:1180-1188.

The structure of mouse KOR-3 gene has been defined as having five exons separated by four introns. Pan et al. (1996). Although structurally homologous with the cloned traditional opioid receptors, mKOR-3 has low affinity for most opioids and opioid peptides. The ligand for KOR-3 has been identified and designated orphanin FQ/nociceptin (OFQ/N). Reinscheid et al. (1995) Science 270:792-794; and Meunier et al. (1995) Nature 377:532-535. OFQ/N is intimately involved with pain perception, but its actions are complex. Initially, it was reported to be hyperalgesic and that low doses reverse the actions of opioids. Reinscheid et al. (1995); Meunier et al. (1995); Mogil et al. (1996) Neurosci. Lett. 214:1-4; Mogil et al. (1996) Neurosci. 75:333-337; Grisel et al. (1996) NeuroReport 7:2125-2129; Tian et al. (1997) Pharmacol. 120:676-680; Zhu et al. (1997) Neurosci. Lett. 235:37-40; and King et al. (1998) Biochem. Pharmacol. 55:1537-1540. Yet OFQ/N is also an analgesic. Tian (1997); Rossi et al. (1997) J. Pharmacol. Exp. Ther. 282:858-865; Rossi et al. (1996) Eur. J. Pharmacol. 31:R7-R8; King et al. (1997) Neurosci. Lett. 223:113-116; and Yamamoto et al. (1997) Neurosci. 81:249-254.

The complex pharmacology of OFQ/N and antisense studies of KOR-3 raised the possibility of multiple OFQ/N receptors. Rossi et al. (1997); Rossi et al. (1996); King et al. (1997); Mathis et al. (1997) Biochem. Biophys. Res. Commun. 230:462-465; and Pan et al. (1995). Radiolabeled OFQ/N binding to brain homogenates is quite distinct from that to the cloned receptor and is consistent with binding site heterogeneity in the brain. Mathis et al. (1997); Reinscheid et al. (1995); and Pan et al. (1996) FEBS Lett. 395:207-210. Antisense mapping of the three coding exons of the receptor encoded by KOR-3

also raised the question of alternative splicing. Pan et al. (1994) Regul. Pept. 54:217-218; Pan et al. (1995); Rossi et al. (1997); Rossi et al. (1997); and Pasternak et al. (1995) Trends Pharmacol. Sci. 16:344-350. Antisense probes targeting the first coding exon blocked OFQ/N hyperalgesia, but not analgesia, whole other probes targeting the second and third exons blocked analgesia and not hyperalgesia. The second and third coding exons, but not the first, also have been implicated in kappa$_3$ analgesia. These observations raised the possibility that the kappa$_3$ receptor and KOR-3 might result from alternative splicing of the same gene. Pan et al. (1996).

Two alternative splice KOR variants have been reported, including a rat variant (XOR1L) which contains a 28 amino acid residue insertion between 15 base deletion corresponding to Tyr71-Arg75 in the first intracellular loop. Wang et al. (1994) FEBS Lett 1994 348:75-79; and Halford et al. (1995) J. Neuroimmunol. 59:91-101.

Availability of polynucleotide sequences encoding opioid receptor splice variants, and the corresponding polypeptide sequences, will significantly increase the capability to design pharmaceutical compositions, such as analgesics, with enhanced specificity of function. In general, the availability of these polypeptide sequences will enable efficient screening of candidate compositions. The principle in operation through the screening process is straightforward: natural agonists and antagonists bind to cell-surface receptors and channels to produce physiologic effects; certain other molecules can produce physiologic effects and act as therapeutic pharmaceutical agents. Thus, the ability of candidate drugs to bind to opioid receptor splice variants can function as an extremely effective screening criterion for the selection of pharmaceutical compositions with a desired functional efficacy and specificity.

DISCLOSURE OF THE INVENTION

The invention encompasses KOR-3 splice variant polypeptides.

The invention further encompasses a KOR-3 splice variant polynucleotide, including those encoding KOR-3 splice variant polypeptides.

The invention further encompasses methods of screening compositions for an opioid activity by obtaining a control cell that does not express a recombinant or endogenous opioid receptor, obtaining a test cell that expresses a recombinant KOR-3 splice variant polypeptide, contacting the control cell and test cell with an amount of an opioid sufficient to exert a physiologic effect, separately measuring the physiologic effect of the composition on the control cell and test cell and comparing the physiologic effect of the composition to the physiologic effect of the opioid, where determination of a physiologic effect of the composition is expressed relative to that of the opioid.

The invention further encompasses methods of screening compositions for an opioid activity by obtaining a control polypeptide that is not a recombinant opioid receptor, obtaining a test polypeptide that is a recombinant KOR-3 splice variant polypeptide, contacting a composition with the control polypeptide and the test polypeptide, contacting the test polypeptide with an amount of an opioid sufficient to measurably bind the test polypeptide, measuring the binding of the composition and the opioid, and comparing the test polypeptide binding of the composition to that of the opioid, where determination of binding of the composition is expressed relative to that of the opioid.

The invention further encompasses methods of screening compositions for differential opioid activity comprising obtaining a first and second test polypeptide that are KOR-3 splice variant polypeptides and contacting each with a composition, measuring the binding affinity of the composition to the first and second test polypeptides and comparing the binding of the composition and the first test polypeptide to that of the second test polypeptide where differential activity is expressed as a ratio of the two binding affinities.

The invention further encompasses a non-human animal in which one or both endogenous KOR-3 alleles has been altered by homologous recombination with an exogenously introduced nucleic acid provided herein.

The invention further encompasses a non-human transgenic animal carrying a transgene comprising a KOR-3 splice variant polynucleotide.

The invention further encompasses a method for regulating morphine analgesia in a subject by altering the amount of KOR-3 polypeptide activity. Activity can be regulated by administering antigen binding fragments, agonists, antagonists or small molecule ligands to a subject in an amount of and a duration sufficient to regulate morphine analgesia. The antigen binding fragment, agonist, antagonist or small molecule ligand is directed to an KOR-3 splice variant polypeptide.

Opioid activity can also be regulated by administering a DNA plasmid vector containing a KOR-3 splice variant polynucleotide. The DNA plasmid vector thereby expresses an KOR-3 polynucleotide in a subject in an amount of and a duration sufficient to regulate morphine analgesia. Activity can also be regulated by administering an antisense nucleic acid complementary to a KOR-3 splice variant polynucleotide, thereby blocking gene expression in a subject in an amount of and a duration sufficient to regulate morphine analgesia.

The invention further encompasses a method for regulating body weight in a subject by altering the amount of KOR-3 polypeptide activity. Activity can be regulated by administering antigen binding fragments, agonists, antagonists or small molecule ligands to a subject in an amount of and a duration sufficient to regulate body weight. The antigen binding fragment, agonist, antagonist or small molecule ligand is directed to an KOR-3 splice variant polypeptide.

Activity can also be regulated by administering a DNA plasmid vector containing a KOR-3 splice variant polynucleotide. The DNA plasmid vector thereby expresses an KOR-3 polynucleotide in a subject in an amount of and a duration sufficient to regulate body weight. Activity can also be regulated by administering an antisense nucleic acid complementary to a KOR-3 splice variant polynucleotide, thereby blocking gene expression in a subject in an amount of and a duration sufficient to regulate body weight.

The invention further encompasses a method for diagnosing an KOR-3 splice variant-associated pharmacological abnormality, comprising measuring the amount of variant activity or tissue distribution thereof in a subject and comparing that activity or tissue distribution to a control sample, wherein a difference in the

amount of activity or tissue distribution correlates with the presence of a pharmacological defect.

The invention further encompasses a method for diagnosing an KOR-3 splice variant-associated disorder of the neuroendocrine system, comprising measuring the amount of variant activity or tissue distribution thereof in a subject and comparing that activity or tissue distribution to a control sample, wherein a difference in the amount of activity or tissue distribution correlates with the presence of a disorder of the neuroendocrine system.

The invention further encompasses antigen-binding fragments specific for the KOR-3 splice variants described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram of KOR-3 genes and alternative splicing. Exons and introns are indicated by open boxes and horizontal lines, respectively. Mini-exon 1c (34 bp) is shown by the solid box with horizontal lines, mini-exon 1b (98 bp) by the solid box with diagonal lines and mini-exon 1c (139 bp) (including mini-exon 1b) by a solid black box and box 1b. The shaded area of exon 1 represents the deletion originally reported in lymphocytes by Halford et al. (1995) which has also now been found in brain. Arrows represent the primers used in RT-PCR to clone the splice variants. The short heavy line over exon 3 denotes the probe used in Southern blotting to detect expression of KOR-3 gene splicing variants.

Figure 2 is the amino acid (AA) sequence specific to mKOR-3A (exons 1,1a,2,3) and the GenBank accession number.

Figure 3 is the AA sequence specific to mKOR-3B (exons 1,1b) and the GenBank accession number.

Figure 4 is the AA sequence specific to mKOR-3C (exons 1,1c) and the GenBank accession number.

Figure 5 is the AA sequence specific to mKOR-3E (exons 1,2,2a).

Figure 6 is the AA sequence specific to rKOR-3A (exons 1,1a,2,3).

Figure 7 is the AA sequence specific to hKOR-3A (exons 1, 1a, 2, 3).

Figure 8 is the AA sequence specific to hKOR 3D (exons 1a, 2, 3).

Figure 9 is the nucleic acid sequence specific to mKOR-3A (exons 1,1a,2,3) and the GenBank accession number.

Figure 10 is the nucleic acid sequence mKOR-3B (exons 1,2,1b,3,4) and the GenBank accession number.

Figure 11 is the nucleic acid sequence mKOR-3C (exons 1,2,1c,3,4,5) and the GenBank accession number.

Figure 12 is the nucleic acid sequence of mKOR-3E (exons 1,2,3,2a,4).

Figure 13 is the nucleic acid sequence specific to rKOR-3A (exons 1,1a,2,3).

Figure 14 is the nucleic acid sequence of hKOR-3A (exons 1,1a,2,3).

Figure 15 is the nucleic acid sequence hKOR-3D (exons 1a,2,3).

Figure 16 is the amino acid alignment of mKOR-3D and hKOR-3D. Dots indicate identities in the sequences; differences are indicated by the indicated residues. Underlined sequences are putative transmembrane regions.

Figure 17 is the amino acid alignment of mKOR-A, rKOR-3A and hKOR-3A. Dots indicate identities in the sequences; differences are indicated by the indicated residues. Bold sequences indicate the predicted amino acid sequence from coding exon 1 and insertion sequences. Underlined sequences are putative transmembrane sequences.

Figure 18 is the insertion sequences of KOR-3, A, B, C and E. Insertion sequences of (a) KOR-3A, (b) KOR-3B and KOR-3C, (c) KOR-3E. Intronic sequences are in lower case and exons are in upper case. Base numbers for genomic and cDNA sequences are based upon intron 2 of the KOR-3 gene (GenBank accession number U32939) and KOR-3 cDNA (GenBank accession number U09421) sequences. The splice junctions are indicated by arrows. In (b) arrows indicate the splice junctions for KOR-3c and KOR-b.

Figure 19 is a Northern blot depicting KOR-3 and its splice variants. The probes used are indicated in the figure.

Figure 20 is a Southern blot depicting the regional distribution of KOR-3 splice variants (KOR-3c, KOR-3a, KOR -3 and KOR-3d) in cortex, cerebellum, striatum, hypothalamus, brain stem, PAG, thalamus, spinal cord and whole brain.

BEST MODE FOR CARRYING OUT THE INVENTION

In view of the strong pharmacological evidence for distinct kappa$_3$-opioid receptors, alternative splicing of the KOR-3 gene has been explored further. It has now been determined that the KOR-3 gene is subject to alternative splicing that produces novel splice variant forms of the receptor. Four new exons for the KOR-3 gene have been identified, which combine to yield seven novel KOR-3 splice variants of mouse, rat and human origin. These splice variants are potential targets for modulating morphine analgesia and opioid-mediated ingestive responses.

This invention encompasses splice variants of the KOR-3 gene exemplified by newly isolated KOR-3 splice variant polypeptides. The exemplary KOR-3 splice variant polypeptides are composed of the amino acids indicated in Figures 2-8. Polypeptides comprising 5 amino acids, more preferably 7 amino acids, more preferably 15 amino acids, more preferably 25 amino acids, more preferably 50 amino acids and more preferably 75 amino acids, which are not the same as known variants are also included. The exemplary KOR-3 splice variant polypeptides retain KOR-3 activity. The complete cDNA sequences of KOR-3A, KOR-3B, and KOR-3C have been deposited in GenBank, numbers AF043276, AF043277, and AF043278 respectively, in satisfaction of the requirements of the Budapest Treaty.

The terms "protein", "peptide", "polypeptide" and "polypeptide fragment" are used interchangeably herein to refer to polymers of amino acid residues of any length. The polymer can be linear or branched, it may comprise modified amino acids or amino acid analogs, and it may be interrupted by chemical moieties other than amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example disulfide bond formation,

glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation of modification, such as conjugation with a labeling or bioactive component.

The KOR-3 splice variant polypeptides, including polypeptide fragments, homologs thereof, retain KOR-3 activity. To "retain KOR-3 activity" is to have a similar level of functional activity as the KOR-3 polypeptide. This activity includes but is not limited to, immunologic and pharmacological activity.

The "immunologic activity" is binding to anti-opioid receptor antigen binding fragments. The antigen binding fragments can be whole native antibodies, bispecific antibodies, chimeric antibodies, Fab, F(ab')2, single chain V region fragments (scFv), and fusion polypeptides comprising an antigen binding fragment fused to a chemically functional moiety.

The "pharmacologic activity" is activation or deactivation of the KOR-3 splice variant polypeptides upon binding of agonists or antagonists.

The invention further encompasses KOR-3 splice variant polypeptide homologs. A "homolog" is a polypeptide similar in amino acid sequence to other polypeptides among a single species or, a "homolog" in evolution is a polypeptide similar in amino acid sequence to other polypeptides in different species because they have been inherited from a common ancestor. Preferably, homologs of the present invention are human homologs.

Isolation of KOR-3 splice variant human homolog cDNAs can be carried out by any method known in the art. For instance, methods analogous to the isolation of the mouse KOR-3 splice variants described herein (see Example 1). Using primers corresponding to the human KOR-3 gene and a Marathon-Ready human cDNA Library to carry out reactions according to the Marathon cDNA Amplification Kit (Clontech), human KOR-3 splice variants can be obtained. Alternatively, screening of human cDNA libraries with probes corresponding to mouse KOR-3 splice variant sequences can be carried out at reduced stringency to identify human KOR-3 splice variant cDNAs.

The invention further encompasses the KOR-3 splice variant polypeptides in a heterodimeric or homodimeric form. A "heterodimer" is a protein made up of

more than one kind of polypeptide. A "homodimer" is a protein made up of more than one kind of polypeptide.

The invention further encompasses isolated KOR-3 splice variant polynucleotide sequences indicated in Figures 9-15 and 18. In addition to these, the polynucleotide sequence can be any sequence of the appropriate genetic code to encode any of the KOR-3 splice variant polypeptides indicated in Figures 2-8, 16 and 17. Preferably, the polynucleotide is at least 15 consecutive nucleotides.

A "polynucleotide" or "nucleic acid" is a polymeric form of nucleotides of any length, which contain deoxyribonucleotides, ribonucleotides, and analogs in any combination. Polynucleotides may have three-dimensional structure, and may perform any function, known or unknown. The term "polynucleotide" includes double-, single-stranded, and triple-helical molecules. Unless otherwise specified or required, any embodiment of the invention described herein that is a polynucleotide encompasses both the double stranded form and each of two complementary forms known or predicted to make up the double stranded form of either the DNA, RNA or hybrid molecule.

The following are non-limiting examples of polynucleotides: a gene or gene fragment, exons, introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes and primers. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs, uracyl, other sugars and linking groups such as fluororibose and thiolate, and nucleotide branches. The sequence of nucleotides may be further modified after polymerization, such as by conjugation, with a labeling component. Other types of modifications included in this definition are caps, substitution of one or more of the naturally occurring nucleotides with an analog, and introduction of means for attaching the polynucleotide to proteins, metal ions, labeling components, other polynucleotides or solid support.

An "isolated" polynucleotide or polypeptide is one that is substantially free of the materials with which it is associated in its native environment. By

substantially free, is meant at least 50%, preferably at least 70%, more preferably at least 80%, and even more preferably at least 90% free of these materials.

The invention further comprises a complementary strand to the polynucleotide encoding the KOR-3 splice variant polypeptide.

The complementary strand may be a polymeric form of nucleotides of any length, which contain deoxyribonucleotides, ribonucleotides, and analogs in any combination.

Hybridization reactions can be performed under conditions of different "stringency". Conditions that increase stringency of a hybridization reaction are well known. See for examples, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook et al. 1989). Examples of relevant conditions include (in order of increasing stringency): incubation temperatures of 25°C, 37 °C, 50 °C, and 68 °C; buffer concentrations of 10 x SSC, 6 x SSC, 1 x SSC, 0.1 x SSC (where SSC is 0.15 M NaCl and 15 mM citrate buffer) and their equivalent using other buffer systems; formamide concentrations of 0%, 25%, 50%, and 75%; incubation times from 5 minutes to 24 hours; 1, 2 or more washing steps; wash incubation times of 1, 2, or 15 minutes; and wash solutions of 6 x SSC, 1 x SSC, 0.1 x SSC, or deionized water.

The invention further encompasses polynucleotides encoding functionally equivalent variants and derivatives of the KOR-3 splice variant polypeptides and functionally equivalent fragments thereof which may enhance, decrease or not significantly affect properties of the polypeptides encoded thereby. These functionally equivalent variants, derivatives, and fragments display the ability to retain KOR-3 activity. For instance, changes in a DNA sequence that do not change the encoded amino acid sequence, as well as those that result in conservative substitutions of amino acid residues, one or a few amino acid deletions or additions, and substitution of amino acid residues by amino acid analogs are those which will not significantly affect properties of the encoded polypeptide. Conservative amino acid substitutions are glycine/alanine; valine/isoleucine/leucine; asparagine/glutamine; aspartic acid/glutamic acid;

serine/threonine/methionine; lysine/arginine; and phenylalanine/tyrosine/tryptophan.

The invention further encompasses the KOR-3 splice variant polynucleotide sequences contained in a vector molecule or an expression vector and operably linked to a promoter element if necessary.

A "vector" refers to a recombinant DNA or RNA plasmid or virus that comprises a heterologous polynucleotide to be delivered to a target cell, either in vitro or in vivo. The heterologous polynucleotide can comprise a sequence of interest for purposes of therapy, and can be in the form of an expression cassette. As used herein, a vector need not be capable of replication in the ultimate target cell or subject. The term includes cloning vectors for translation of a polynucleotide encoding sequence. Also included are viral vectors.

The term "recombinant" means a polynucleotide of genomic cDNA, semisynthetic, or synthetic origin which either does not occur in nature or is linked to another polynucleotide in an arrangement not found in nature.

"Heterologous" means derived from a genetically distinct entity from the rest of the entity to which it is being compared. For example, a polynucleotide, may be placed by genetic engineering techniques into a plasmid or vector derived from a different source, and is a heterologous polynucleotide. A promoter removed from its native coding sequence and operatively linked to a coding sequence other than the native sequence is a heterologous promoter.

The polynucleotides or nucleic acids of the invention can comprise additional sequences, such as additional encoding sequences within the same transcription unit, controlling elements such as promoters, ribosome binding sites, polyadenylation sites, additional transcription units under control of the same or a different promoter, sequences that permit cloning, expression, homologous recombination, and transformation of a host cell, and any such construct as may be desirable to provide embodiments of this invention.

A "host cell" denotes a prokaryotic or eukaryotic cell that has been genetically altered, or is capable of being genetically altered by administration of an exogenous polynucleotide, such as a recombinant plasmid or vector. When

referring to genetically altered cells, the term refers both to the originally altered cell, and to the progeny thereof.

Polynucleotides comprising a desired sequence can be inserted into a suitable cloning or expression vector, and the vector in turn can be introduced into a suitable host cell for replication and amplification. Polynucleotides can be introduced into host cells by any means known in the art. The vectors containing the polynucleotides of interest can be introduced into the host cell by any of a number of appropriate means, including direct uptake, endocytosis, transfection, f-mating, electroporation, transfection employing calcium chloride, rubidium chloride, calcium phosphate, DEAE-dextran, or other substances; microprojectile bombardment; lipofection; and infection (where the vector is infectious, for instance, a retroviral vector). The choice of introducing vectors or polynucleotides will often depend on features of the host cell.

Once introduced, the exogenous polynucleotide can be maintained within the cell as a non-integrated vector (such as a plasmid) or integrated into the host cell genome. Amplified DNA can be isolated from the host cell by standard methods. See, e.g., Sambrook et al. (1989). RNA can also be obtained from transformed host cell, or it can be obtained directly from the DNA by using a DNA-dependent RNA polymerase.

Expression vectors generally are replicable polynucleotide constructs that contain a polynucleotide encoding the polypeptide of interest. Herein, this means any of the KOR-3 splice variant polypeptides. For expression, one or more translational controlling elements are also usually required, such as ribosome binding sites, translation initiation sites and stop codons. These controlling elements (transcriptional and translational) can be derived from the KOR-3 gene, or heterologous (i.e., derived from other genes or other organisms). A number of expression vectors suitable for expression in eukaryotic cells including yeast, avian, and mammalian cells are well known in the art. One example of an expression vector is pcDNA3 (Invitrogen, San Diego, CA), in which transcription is driven by the cytomegalovirus (CMV) early promoter/enhancer. This vector also contains recognition sites for multiple restriction enzymes for insertion of an

KOR-3 splice variant polypeptide of interest. Another example of an expression vector system is the baculovirus/insect system.

Cloning and expression vectors typically contain a selectable marker (for example, a gene encoding a protein necessary for the survival or growth of a host cell transformed with the vector), although such a marker gene can be carried on another polynucleotide sequence co-introduced into the host cell. Only those host cells into which a selectable gene has been introduced will grow under selective conditions. Typical selection genes either: (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate; (b) complement auxotrophic deficiencies; or (c) supply critical nutrients not available for complex media. The choice of the proper marker gene will depend on the host cell, and appropriate genes for different hosts are known in the art. Vectors also typically contain a replication system recognized by the host.

Suitable cloning vectors can be constructed according to standard techniques, or selected from a large number of cloning vectors available in the art. While the cloning vector selected may vary according to the host cell intended to be used, useful cloning vectors will generally have the ability to self-replicate, may possess a single target for a particular restriction endonuclease, or may carry marker genes. Suitable examples include plasmids and bacterial viruses, e.g., pUC18, mp18, mp19, pBR322, pMB9, ColE1, pCR1, RP4, phage DNAs, and shuttle vectors such as pSA3 and pAT28. These and other cloning vectors are available from commercial vendors such as BioRad, Stratagene, and Invitrogen.

The invention further encompasses non-human animals in which one or both KOR-3 alleles has been altered by homologous recombination with an exogenously introduced nucleic acid.

Non-human animals devoid of one or more gene products are generated to determine the "loss-of-function" phenotype associated with the loss of that particular gene product. Herein, the gene product is the KOR-3 gene or splice variants thereof. Phenotypic abnormalities can be present, for instance, in anatomical structures, biochemical and genetic pathways and pharmacological

responses. Loss-of-function phenotypic analysis has the potential to reveal the function of the gene product.

Methods of homologous recombination with an exogenously introduced nucleic acid are used to inactivate one or more alleles in non-human animals. These methods, as applied to mice and rats, are well known in the art. Capecchi (1989) Science 244:1288. Usually, an exogenous polynucleotide encoding a selectable marker gene, and having sufficient sequence homology to the targeted site of integration at either end of the polynucleotide, is introduced into the genome of embryonic stem cells (ES cells) derived from the inner cell mass of non-human animal blastocysts. Evans and Kaufman (1981) Nature 292:154. Through homologous recombination, the polynucleotide is incorporated into the genetic locus at the targeted site of integration, replacing the corresponding sequences of the endogenous allele. ES cells are used to generate chimeric animals either by microinjection into, or aggregation with wildtype embryos. Chimeric animals having germ line transmission of the inactivated allele are bred to produce heterozygous, and subsequently, homozygous lines carrying the inactivated allele. Robertson (1991) Biol. Reprod. 44:238.

The invention further encompasses non-human transgenic animals carrying a transgene encoding an KOR-3 splice variant polypeptide.

Non-human animals carrying additional copies of the gene of interest are generated to determine the "gain-of-function" phenotype associated with excess of that particular gene product. Herein, the gene product is any of the KOR-3 gene splice variants. Phenotypic abnormalities can be present, for instance, in anatomical structures, biochemical and genetic pathways and pharmacological responses. Gain-of-function phenotypic analysis has the potential to reveal the function of the gene product.

Methods of generating transgenic animals are well known in the art. Jaenisch (1988) Science 240:1468. "Transgenes" are exogenous polynucleotides encoding the gene of interest. Transgenes are introduced into the embryonic genome through microinjection. Alternatively, a transgene encoding the gene of interest and a selectable marker gene is introduced into the ES cell genome

through transfection or electroporation. ES cells carrying the transgene are subsequently used to produce animals with multiple copies of the gene of interest.

Pharmaceutical compositions and treatment modalities can be detected by the methods of this invention. The KOR-3 splice variant polypeptides and corresponding nucleic acid sequences can be used in screening for compositions that alter variant activity. Compositions that selectively regulate the KOR-3 splice variant polypeptides or selectively modulate physiologic processes can be identified.

The invention further encompasses methods of screening compositions for opioid activity by obtaining a control cell that does not express a recombinant opioid receptor and obtaining a test cell that is the same as the control cell except that it expresses a recombinant KOR-3 splice variant polypeptide, contacting the control cell and test cell with an amount of an opioid sufficient to exert a physiologic effect, separately measuring the physiologic effect of the composition on the control cell and test cell and comparing the physiologic effect of the composition to the physiologic effect of the opioid, where determination of a physiologic effect of the composition is expressed relative to that of the opioid.

The invention further comprises a method of screening compositions for opioid activity by obtaining a control polypeptide that is not a recombinant opioid receptor and obtaining a test polypeptide that is a recombinant KOR-3 splice variant polypeptide, contacting a composition with the control polypeptide and the test polypeptide, contacting the test polypeptide with an amount of an opioid sufficient to measurably bind the test polypeptide, measuring the binding of the composition and the opioid and comparing the test polypeptide binding of the composition to that of the opioid, where determination of binding of the composition is expressed relative to that of the opioid.

The invention further encompasses a method of screening compositions for differential opioid activity by obtaining a first test polypeptide that is a KOR-3 splice variant polypeptide, and contacting it with a composition and obtaining a second test polypeptide that is an KOR-3 splice variant polypeptide, measuring the binding of the composition to the first and second test polypeptides, and

comparing the binding of the composition and the first test polypeptide to that of the second test polypeptide where differential activity is expressed as a ratio of the two binding affinities.

The compositions screened include but are not limited to chemical, synthetic combinatorial libraries of small molecule ligands, eukaryotic whole cell lysates or extracts, media conditioned by cultured eukaryotic cells, natural products and extracts thereof.

The opioid can be but is not limited to, morphine, etorphine, levorphanol, [D-Ala^2, MePhe^4, Gly(ol)^5]enkephalin (DAMGO), pentazocine, nalbuphine, naloxone benzoylhydrazone, ethylketocyclazocine, bremazocine and analogs thereof.

The physiologic effect can be measured by any method known in the art such as changes in the levels of neuroendocrine hormones including but not limited to prolactin, growth hormone, gonadotropin-releasing hormone, adrenocorticotropin, corticotropin-releasing factor, luteinizing hormone, follicle stimulating hormone, testosterone or cortisol.

The physiologic effect is also measured by changes in the levels of neurotransmitters, including but not limited to, acetylcholine or dopamine.

Activation of the KOR-3 receptor, and likely, the KOR-3 splice variant polypeptides, stimulates a variety of physiologic responses, including analgesia, depression of gastrointestinal motility and respiration, and alterations of the endocrine and autonomic nervous system. Compositions that regulate the activity of the KOR-3 receptor and/or the KOR-3 splice variant polypeptides can elicit responses that have therapeutic effects. The invention is useful in diagnosis, treatment, design and screening of novel reagents.

The invention further encompasses a method for regulating morphine analgesia in a subject by altering the amount of KOR-3 polypeptide activity in the subject. Activity can be regulated by administering antigen binding fragments, agonists, antagonists or small molecule ligands to a subject in an amount and a duration sufficient to regulate morphine analgesia. The antigen binding fragment,

agonist, antagonist or small molecule ligand is directed to an KOR-3 splice variant polypeptide.

Activity can also be regulated by administering a DNA plasmid vector containing a KOR-3 splice variant polynucleotide. The DNA plasmid vector thereby expresses an KOR-3 polypeptide in a subject in an amount and a duration sufficient to regulate morphine analgesia. Activity can also be regulated by administering an antisense nucleic acid complementary to a KOR-3 splice variant polynucleotide, thereby blocking gene expression in a subject in an amount and a duration sufficient to regulate morphine analgesia.

The invention further encompasses a method for regulating body weight in a subject by altering the amount of KOR-3 polypeptide activity. Activity can be regulated by administering antigen binding fragments, agonists, antagonists or small molecule ligands to a subject in an amount and a duration sufficient to regulate body weight. The antigen binding fragment, agonist, antagonist or small molecule ligand is directed to a KOR-3 splice variant polypeptide.

Activity can also be regulated by administering a DNA plasmid vector containing a KOR-3 splice variant polynucleotide. The DNA plasmid vector thereby expresses a KOR-3 polynucleotide in a subject in an amount of and a duration sufficient to body weight. Activity can also be regulated by administering an antisense nucleic acid complementary to a KOR-3 splice variant polynucleotide, thereby blocking gene expression in a subject in an amount and a duration sufficient to regulate body weight.

Agonists and antagonists of KOR-3 splice variant activity can include but are not limited to, morphine, methadone, etorphine, levorphanol, fentanyl, sufentanil, [D-Ala^2, MePhe^4, Gly(ol)5]enkephalin (DAMGO), butorphanol, naloxone, naltrexone, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP), diprenorphine, β-funaltrexamine, naloxonazine, nalorphine, pentazocine, nalbuphine, benzoylhydrazone, bremazocine, ethylketocyclazocine, U50488, U69593, spiradoline, naltrindole, [D-Pen^2, D-Pen-^{5}]enkephalin (DPDPE), [D-Ala^2,Glu^4]deltorphin, [D-Ser^2,Leu^5]enkephalin-Thr^6 (DSLET), Met-enkephalin, Leu-enkephalin, β-endorphin, dynorphin A, dynorphin B, α-neoendorphin.

A "subject" is a vertebrate, preferably a mammal, and more preferably a human. Mammals include but are not limited to humans, farm animals, sport animals, and pets.

The invention further encompasses a method for diagnosing a KOR-3 splice variant-associated pharmacological abnormality, comprising measuring the amount of variant activity or tissue distribution thereof in a subject and comparing that activity or tissue distribution to a control sample, wherein a difference in the amount of activity or tissue distribution correlates with the presence of a pharmacological defect. This disorder can be heritable.

The invention further encompasses a method for diagnosing a KOR-3 splice variant-associated disorder of the neuroendocrine system, comprising measuring the amount of variant activity or tissue distribution thereof in a subject and comparing that activity or tissue distribution to a control sample, wherein a difference in the amount of activity or tissue distribution correlates with the presence of a disorder of the neuroendocrine system. This disorder can be heritable.

The invention further encompasses methods for generating antigen binding fragments specific for a KOR-3 splice variant polypeptide. According to the invention, a KOR-3 splice variant polypeptide can be used as an immunogen to generate antigen binding fragments which immunospecifically bind the immunogen.

Production of antigen binding fragments such as polyclonal antibodies may be carried out by any method known in the art. Various host animals can be immunized by injection with the immunogen, including but not limited to rabbits, mice and rats. Various adjuvants can be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete or incomplete) adjuvant, mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin) and Corynebacterium parvum.

For preparation of antigen binding fragments such as monoclonal antibodies, any technique which provides for the production of antibody molecules by continuous cell lines in culture can be used. Examples of such techniques include the original hybridoma technique (Kohler and Milstein (1975) Nature 256:495) as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al. (1983) Immunol. Today 4:72), and the EBV hybridoma technique to produce human monoclonal antibodies (Cole et al. (1985) in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Monoclonal antibodies can also be produced in germ-free animals utilizing recent technology (PCT/US90/02545). Human antibodies can be obtained using human hybridomas (Cote et al. (1983) Proc. Natl. Acad. Sci. U.S.A. 80:2026), or by transforming human B cells with EBV virus in vitro (Cole et al. (1985) in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Techniques developed for the production of "chimeric antibodies" (Morrison et al. (1984) Proc. Natl. Acad. Sci. U.S.A. 81:6851; Neuberger et al. (1984) Nature 312:604; and Takeda et al. (1985) Nature 314:452) by splicing the genes from a mouse antibody molecule specific for KOR-3 splice variants together with genes from a human antibody of appropriate bioligical activity can be used.

Techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce KOR-3 splice variant-specific single chain antibodies. Techniques described for the production of Fab expression libraries (Huse et al. (1989) Science 246:1275) can be utilized, allowing rapid and easy identification of monoclonal Fab fragments specific for a KOR-3 splice variant polypeptide.

Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the F(abl), fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the F(abl) fragment, the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent, and Fv fragments.

Single chain V region fragments ("scFv") can also be produced. Single chain V region fragments are made by linking L (light) and/or H (heavy) chain V (variable) regions by using a short linking peptide. Bird et al. (1988) Science 242:423. Any peptide having sufficient flexibility and length can be used as a linker in a scFv. Usually the linker is selected to have little to no immunogenicity. An example of a linking peptide is $(GGGGS)_3$, which bridges approximately 3.5 nm between the carboxy terminus of one V region and the amino terminus of another V region. Other linker sequences can also be used, and can provide additional functions, such as a means for attaching a drug or a solid support.

All or any portion of the H or L chain can be used in any combination. Typically, the entire V regions are included in the scFv. For instance, the L chain V region can be linked to the H chain V region. Alternatively, a portion of the L chain V region can be linked to the H chain V region, or a portion thereof. Also contemplated are scFvs in which the H chain V region is from H11, and the L chain V region is from another immunoglobulin. It is also possible to construct a biphasic, scFv in which one component is a KOR-3 splice variant polypeptide and another component is a different polypeptide, such as a T cell epitope.

The scFvs can be assembled in any order, for example, V_H—(linker)—V_L or V_L—(linker)—V_H. There may be a difference in the level of expression of these two configurations in particular expression systems, in which case one of these forms may be preferred. Tandem scFvs can also be made, such as (X)—(linker)—(X)—(linker)—(X), in which X are KOR-3 splice variant polypeptides, or combinations of KOR-3 splice variant polypeptides with other polypeptides. In another embodiment, single chain antibody polypeptides have no linker polypeptide, or just a short, inflexible linker. Exemplary configurations include V_L—V_H and V_H—V_L. The linkage is too short to permit interaction between V_L and V_H within the chain, and the chains form homodimers with a V_L/V_H antigen binding site at each end. Such molecules are referred to in the art as "diabodies".

ScFvs can be produced either recombinantly or synthetically. For synthetic production of scFv, an automated synthesizer can be used. For recombinant production of scFv, a suitable plasmid containing a polynucleotide that encodes the scFv can be introduced into a suitable host cell, either eukaryotic, such as yeast, plant, insect or mammalian cells, or prokaryotic, such as *Escherichia coli*, and the protein expressed by the polynucleotide can be isolated using standard protein purification techniques.

A particularly useful system for the production of scFvs is plasmid pET-22b(+) (Novagen, Madison, WI) in *E. coli*. pET-22b(+) contains a nickel ion binding domain consisting of 6 sequential histidine residues, which allows the expressed protein to be purified on a suitable affinity resin. Another example of a suitable vector is pcDNA3 (Invitrogen, San Diego, CA), described above.

Expression conditions should ensure that the scFv assumes functional and, preferably, optimal tertiary structure. Depending on the plasmid used (especially the activity of the promoter) and the host cell, it may be necessary to modulate the rate of production. For instance, use of a weaker promoter, or expression at lower temperatures, may be necessary to optimize production of properly folded scFv in prokaryotic systems; or, it may be preferably to express scFv in eukaryotic cells.

The following examples are provided to illustrate but not limit the claimed invention.

Example 1

Materials and Methods

Identification of alternative splicing variants by RT-PCR

A sense (5'-TGCC TTC CTG CCC CTT GGA C-3'; positions 419-438) and an antisense primer (5'-CCC AGA AGG ATG TCT GTG CCC-3'; position 610-630) based upon the nucleotide sequence of the mouse KOR-3 clone (GenBank accession number U09621) were used to amplify cDNA fragments using PCR. The template was first-strand cDNA reverse transcribed with random hexameters from C57BL/6 mouse brain total RNA prepared as described by Chomczynski et al. (1987) Anal. Biochem. 162:156-159. Multiple bands were obtained by PCR, isolated, subcloned and sequenced in both directions. Four KOR-3-related clones were identified.

cDNA library screening

A C57BL/6 mouse brain λZAP cDNA library was screened with a ^{32}P-labeled 1.1 kb fragment containing the full length KOR-3 coding region at high stringency, identifying 32 positives. Three contained a 34 bp insertion (KOR-3a), one had a 98 bp insertion (KOR-3b), one had a 139 bp insertion (KOR-3c) and another had an 81 bp insertion between coding exons 2 and 3 (KOR-3e). KOR-3a and KOR-3c clones of approximately 2.9 kb and a 1.2 kb clone of KOR-3b were sequenced. Clones with the 15 bp deletion in the first exon in these screens were not observed.

Northern blot analysis

Polyadenylated RNAs were isolated from mouse total RNAs using oligo(dT) chromatography (Pharmacia, Piscataway, NJ) as described by Pan et al. (1995) Mol. Pharmacol. 47:1180-1188. Northern blotting followed the protocol for GeneScreen Plus membranes (New England Nuclear, Boston, MA). Probes for KOR-3a (5'-GGT GTG CCT GCT GTC TCC AGT TCC CCT CAA TGC CCT CCC AGC TGA GGA-3') and KOR-3b/KOR-3c (5'-CCT CAG TCT CTC TTA AGA CTC TCA GAG GGT TTT CAG GGC ACT GCC-3') were 5'-end ^{32}P-labeled by T4 polynucleotide kinase. A ^{32}P-labeled 1.1 kb fragment containing the full length of the KOR-3 coding region was generated by PCR with appropriate primers.

Analysis of KOR-3a, KOR-3b and KOR-3c expression in various brain regions

Total RNAs from various C57BL/6 mouse brain regions were extracted and reverse transcribed using random hexameters. Two primers were designed from the nucleotide sequence of mouse KOR-3/ORL-1 receptor at positions 486-505 (sense primer. 5'-TCC TGG GGA ACT GCC TCG TC-3') and 610-630 (antisense primer, 5'-CCC AGA AGG ATG TCT GTG CCC-3') and used in sequential PCR reactions with the first-strand cDNAs as templates. The predicted sizes of the amplified cDNA fragments for KOR-3, KOR-3a, KOR-3b and KOR-3c are 145 bp, 179 bp, 232 bp and 284 bp, respectively. The PCR products were then separated by 1.5% agarose gel, transferred on GeneScreen Plus membranes and hybridized with a ^{32}P-labeled 107 bp fragment of the coding exon 2 generated

by PCR (Fig. 1). The RNA loading in the RT-PCR reactions was estimated by parallel PCR with β_2-microglobulin primers (Clontech).

Example 2

Results

cDNA cloning of alternative splicing variants of KOR-3 gene

To look for variants differing in the region between the first and second coding exons, RT-PCR was performed using an upstream primer in the first coding exon and a downstream primer in the second. This led to the identification of four splice variants. Three had insertions between the first and second coding exons (Fig. 1) while the fourth had a 15 bp deletion at the 3'-end of the first coding exon, corresponding to a variant cloned from lymphocytes. Halford et al (1995).

Full length cDNAs were then isolated containing the 34 bp (KOR-3a), the 98 bp (KOR-3b) and the 139 bp (KOR-3c) insertions by screening a mouse brain cDNA library with a full length KOR-3 probe. The sequences of the new cDNAs were identical to that of the KOR-3 cDNA except for the insertions. The nucleotide sequences obtained and the corresponding amino acid residues encoded thereby are depicted in Figures 2-15. These sequences include human and rat homologs obtained by screening the relevant cDNA libraries and subsequent cloning.

Comparison of mKOR-3D and hKOR-3D are provided in Figure 16, while mKOR-3A, rKOR-3A and hKOR-3A are compared in Figure 17. The insertion sequences of KOR-3a, KOR-3b and KOR-3c were aligned to the intron between the first and second coding exons of the KOR-3 gene (Pan et al (1996)) and the 81 bp insertion in KOR-3e to the intron between the second and third coding exons (Fig. 18). All of the splice sites were in agreement with the GT/AG rule.

Northern analysis of KOR-3 and its splice variants

Northern blot analysis was then used to investigate the full length transcripts of the variants obtained from whole brain mRNAs (Fig. 19). The KOR-3a probe hybridized a major transcript with a size of approximately 2.9 kb,

with another smaller sized band of lower abundance. Since the 139 bp insertion in KOR-3c contains the 98 bp in KOR-3b, there is no specific probe for the KOR-3b. A 48 bp probe derived from the 98 bp was used to detect both the KOR-3b and KOR-3c expression. This probe hybridized a major transcript with a size of approximately 3.4 kb, which was similar to that probed by the KOR-3b probe. The relative abundance of the transcripts revealed by the KOR-3a probe was much higher than that by the KOR-3b and the KOR-3c probe, although it was less abundant than that by the KOR-3 probe. Bands with the KOR-3e probe were not visible.

Regional expression of the splice variants

The regional distribution of the variants using RT-PCR and Southern blotting was next examined. Four major bands were obtained with different intensities among the various regions (Fig. 20). The sizes of the four bands from the lower to the higher matched those of the 15 bp deletion (clone D), KOR-3, 34 bp insertion (clone A) and 139 bp insertion (clone C), respectively. To confirm that the amplified bands correspond to the KOR-3 splice variants, each band was extracted form the agarose gel, subcloned into the Bluescript vector and sequenced. In all cases, the sequences of the bands were identical to those of the variant clones. The relative abundance in whole brain of KOR-3 and its variants was similar to that seen with Northern blotting: KOR-3 > KOR-3a > KOR-3c. Although KOR-3b expression was not seen in the initial PCR and the blotting, the KOR-3b fragment was amplified by a second round PCR using the gel extracts corresponding to the KOR-3b from the first PCR as templates. This implies a very low level of expression of KOR-3b in brain.

Among the regions examined, the KOR-3 transcript was most abundant in the hypothalamus and periaqueductal gray (PAG), while the cortex, striatum and brainstem had higher levels of the KOR-3a. The KOR-3c was highly expressed in PAG and hypothalamus and the KOR-3d in cerebellum, hypothalamus and brainstem. Lower levels of KOR-3c were seen in the cortex, PAG and thalamus. Interestingly, the only major variant expressed in the striatum was KOR-3a. This

differential expression of the variants among the regions implies region-specific splicing.

Example 3

Discussion

In addition to the variants reported earlier, we have now identified a number of additional splice variants of KOR-3/ORL-1. The insertions observed in the new variants correspond to the region between TM1 and the first intracellular loop, a splice site common among all the opioid receptors. Similar splicing variants with insertions between exons one and two have been identified in mouse delta receptors (DOR-1) gene (Gavereiaux-Ruff et al: (1997) Mol. Brain Res. 48:298-304) and the mu (MOR-1) gene. The presence of such a large number of variants underscores the extensive alternative splicing at this location. The splicing becomes even more interesting in view of the differential regional expression of these variants. Perhaps the best example is the striatum, which has been reported to be devoid of ORL-1 message and OFQ/N receptors. Little evidence was found for any appreciable levels of KOR-3/ORL-1 in this region, in contrast to the high expression of KOR-3a.

Identification of multiple KOR-3 variants with insertions between the first and second coding exons implies extensive alternative splicing at this location. G triplet repeats may play a role in exon-intron border selection and in alternative exon determination (McCullough et al. (1997) Mol. Cell. Biol. 17:4562-4571) and the 34 bp insertion sequence in KOR-3a contains three G triplets. Although the role of the G triplets within the 34 bp in KOR-3a splicing has not been established, it may be interesting to determine whether specific factors capable of binding the G triplets are differentially expressed among brain regions. It has also been reported that many alternatively spliced exons contain GAR repeats, where R is a purine, which have been referred to as exonic splicing enhancers of ESEs. Nagel et al. (1998) RNA 4:11-23. SRp55 is a specific ESE binding protein. Thus, the presence of multiple GAR repeats in the insertion sequences of both the KOR-3b and the KOR-3c might contribute to the regional expression of the KOR-3b and the KOR-3c.

The KOR-3e appears to be an intron-retention variant and is similar to a rat variant. Wang et al. (1994) FEBS Lett. 348:75-79. Unlike the murine version, which contains a termination codon which would lead to a truncated receptor lacking the last three transmembrane regions, the published rat version does not appear to have the termination codon.

Translation of the cDNAs using the start codon AUG of KOR-3 results in early termination either at the mini-exon insertion (KOR-3c) or shortly after the insertion (KOR-3a and KOR-3b). Yet, in preliminary studies the expressed full length clones containing these insertions bind the kappa$_3$ ligand [^{3}H]naloxone benzolhydrazone quite well. Although the truncated protein may retain high affinity for the ligands, this seems unlikely.

All references cited herein, are hereby incorporated herein. Although the foregoing invention has been described in some detail, by way of illustration and example for the purposes of clarity and understanding, it will be apparent to those skilled in the art that certain changes and modifications can be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.